SIZE MEMORY OF CASEIN COLLOID PARTICLES

W. L. CHOATE, F. A. HECKMAN,[1] AND T. F. FORD[2]
Eastern Utilization Research and Development Division, USDA, Washington, D. C.

SUMMARY

Fractions of the casein colloid of fresh milk prepared by differential centrifugation, followed by dialysis of each fraction against sodium barbiturate buffer, have been shown by electrophoretic analysis to have the same ratio of α- to β-casein and, by ultracentrifugal analysis, one major component with a sedimentation (S) value of 5.5×10^{-13}. Other components, present in lower concentrations, appear both in the electrophoretic and in the ultracentrifugal patterns. These sodium caseinate preparations, when redialyzed against skimmilk, became opaque and resembled milk. Ultracentrifugal analysis gives S values in the same range as in the original milk. Moreover, the fractions with the larger S values in milk have the larger S values in the reaggregated colloids, ranging from 100 to about 2,000 in the original milk and from 100 to about 400 in the reaggregated colloids.

The state of aggregation of the casein in milk is of interest because of its bearing on milk and milk product stability problems. In its natural state, milk contains casein colloid particles in a range of sizes (1). It is known that substitution of sodium for calcium ion in the casein complex of milk results in reduction of these sizes and the production of a translucent solution of sodium caseinates (8).

The average particle size of the sodium caseinates, as determined by ultracentrifugation, is many times smaller than the average particle size of the calcium caseinate complex existing in the original milk (4).

It is also known (5) that these relatively low molecular weight sodium caseinates aggregate by the readdition of calcium ion to the system. It was felt that the ideal system in which to study this reaggregation would be one containing all of the components of skimmilk except calcium caseinate. Such a system was here nearly obtained by dialysis of sodium caseinates against relatively large volumes of fresh skimmilk.

The purpose of this study was to determine whether the average particle size of the original colloid fraction affected the sizes of aggregates produced by the final redialysis against skimmilk.

METHOD AND MATERIALS

The general procedure used was as follows: A single skimmilk sample was fractionated, in a preparative centrifuge, into a series of casein colloid fractions of various sizes, represented by average S values (where $S = s \times 10^{-13}$ cm/sec per unit centrifugal field), ranging from 180 to 1,850.[3] Each fraction was then

[1] Present address: W. R. Grace and Co., Washington Research Center, Clarkesville, Maryland.

[2] Present address: Naval Research Laboratory, Washington, D. C.

[3] S values referred to milk, i.e., corrected to the viscosity of skimmilk at the concentration used. The average viscosity of the starting skimmilk was 1.70, relative to water at 20° C.

dialyzed against sodium barbiturate buffer, to replace the bound calcium with sodium. This sodium caseinate preparation was then dialyzed against fresh skimmilk to bring about reaggregation. Thus, there were three states of aggregation possible for each fraction: *(1)* the initial state as present in raw milk; *(2)* the state after dissociation by sodium barbiturate dialysis, and *(3)* the state after reaggregation by dialysis against skimmilk. The effect of each treatment was studied by ultracentrifugal analysis.

Bowl rotor fractionation of the casein colloid. The casein colloid of milk was fractionated by differential centrifugation as follows: A milk sample, collected from a single animal, was centrifuged at a known centrifugal force for 1 min. A small portion of the colloid was thus deposited on a celluloid liner placed in the annulus of the rotor. The rotor was stopped and the deposit removed. The supernatant, now containing most of the particles smaller than those just deposited, was returned to the rotor and the sample centrifuged again, under the same centrifugal force, but for a longer time. In this fashion, a series of deposited fractions containing progressively smaller particles was obtained.

The centrifuge used was an air-driven, air-floated bowl rotor of 70-ml. capacity and 5.5-cm. average radius (2, 3). It was operated at 29,700 r.p.m. for all experimental fractionations.

Data obtained from the fractionation of a typical casein complex from skimmilk with this technique, and the ultracentrifugal analysis of each fraction, are shown in Table 1. Sedimentation constants were determined on 2% solutions of the calcium caseinate colloid. These constants were unchanged after the solutions had been held for seven days at 4° C.

TABLE 1
Bowl rotor fractionation of casein complex from skimmilk at 29,700 r.p.m.

Fraction	Cent. time	Sedimentation constant
	(min.)	*($s_{20,w}$)*
1	1.0	980
2	3.8	870
3	10.8	640
4	60.0	230

Washing of casein colloid fractions. In several experiments, the deposits of casein colloid were washed before dialysis against buffer. In preliminary experiments it was found that washing of the deposit with water produced a more homogeneous fraction, as seen in the ultracentrifuge patterns of the resuspended samples after each washing. Each washing produced a more nearly uniform particle size; however, no further sharpening of the ultracentrifuge peaks was observed after the third washing.

The washing procedure was carried out as follows: Each particular deposit was resuspended in a volume of liquid equal to the volume of skimmilk originally centrifuged. This suspension was then centrifuged for the same time and at the same speed as the skimmilk from which the deposit was obtained. The washings were discarded. Final dissolution of the deposit was accomplished by gently

rotating an Erlenmeyer flask containing the protein, the solvent, and small glass beads for 30 min. at 4° C. A uniform suspension was obtained with no visible precipitate. All centrifugations and washings were done at 20° C.

Washing was done either with distilled water or with milk serum. All of the fractions from one series, however, were given identical treatment; that is, fractions containing particles of different sizes from one series of centrifugations were all washed with water, or were all washed with serum. Milk serum was prepared by centrifugation of a portion of the skimmilk used for micelle fractionation. The centrifuging time for serum preparation was 90 min. The procedure produced a clear, straw-colored solution containing less than 5% of the original casein.

Sodium barbituate dialyses of casein micelles. The casein colloid fractions were dialyzed against sodium barbiturate buffer (pH 8.4, ionic strength 0.10). A sample volume of 20 ml. was dialyzed for 48 hr. against four changes of 1 liter each of buffer at 4° C. in a rotating dialyzer. An aliquot from the dialysis bag of each sample was then analyzed at 59,780 r.p.m. in the ultracentrifuge.

Skimmilk dialysis of sodium caseinate. At the start of ultracentrifugal analysis of each dialyzed sample, an aliquot of the sample was placed in a dialysis bag and dialysis started against fresh skimmilk. Samples were dialyzed against six to ten changes of 2 liters each of skimmilk during six to 16 days. One drop of toluene was placed inside the dialysis bag and one drop in each 2-liter volume of dialysate. Dialysis was at 4° C. At the end of the dialysis, an aliquot of the sample was removed from each dialysis bag and analyzed in the ultracentrifuge (average rotor) at 10,510 r.p.m. To establish the complete conversion of the sodium caseinates to an aggregated form, an aliquot from each sample was also centrifuged at 59,780 r.p.m. and examined for low-molecular weight components. No components in the size range of the unaggregated sodium caseinates were found. To determine whether equilibrium aggregation had been reached, a second aliquot of the aggregated sample was allowed to dialyze against skimmilk for 3 to 7 additional days and again ultracentrifuged. No change was detected.

Ultracentrifugation. The Spinco Model E Ultracentrifuge was used for all sedimentation velocity experiments. Sample temperatures for ultracentrifugation varied between 20 and 25° C. The size range of particles obtained from preparative centrifugation was measured on 2% protein solutions in the solvent used for the washings. A speed of 10,510 r.p.m. was used for these analyses.

The relatively low-molecular-weight sodium caseinates were centrifuged at 59,780 r.p.m. The reaggregated, micelle-like particles resulting from skimmilk dialysis of sodium caseinate were centrifuged at 10,510 r.p.m.

Sedimentation constants were calculated using the increment method (6) and reduced to the viscosity of water or of milk at 20° C.; they were also corrected for adiabatic effects for those experiments carried out at 59,730 r.p.m. (7).

RESULTS AND DISCUSSION

It was found that washed calcium caseinate complex fractions of varying size, prepared by differential centrifugation, were converted by dialysis against

sodium barbiturate buffer to identical solutions of sodium caseinate (as seen in ultracentrifuge patterns), regardless of the size range of the original casein complex micelles (Figure 1). However, when these fractions were reaggregated by dialysis against skimmilk, it was found that the particle sizes of the resulting aggregates were dependent upon the average particle size of the original casein colloid fractions; that is, sodium caseinate made from large casein complex particles aggregated to form large particles, whereas sodium caseinates made from small casein colloid particles aggregated to form small particles when dialyzed against skimmilk. The casein in these colloid fractions retained some sort of "memory" factor or factors causing it to reaggregate to a a predetermined extent in the presence of the dialyzable components of milk.

It was also found that the different casein complex fractions which were not washed, or were washed with milk serum, showed quite different ultracentrifuge patterns after dialysis against sodium barbiturate than those of colloids which were washed with distilled water. Since the washings were not analyzed ultra-

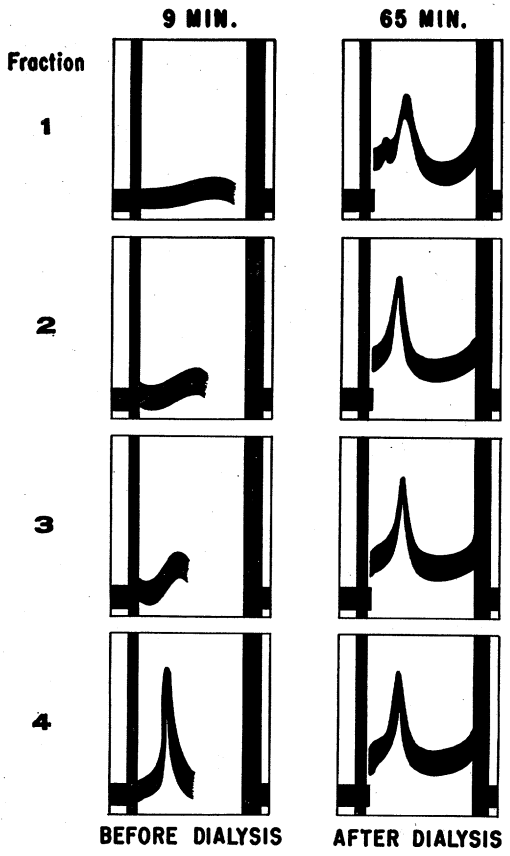

FIG. 1. Ultracentrifuge patterns of centrifugally fractionated cuts of casein colloid from skimmilk; before and after dialysis against sodium barbiturate buffer; sedimentation is from left to right.

centrifugally, it can not be stated whether one component is being converted to another in the sodium caseinate system or whether a micelle protein is being washed out preferentially.

The average sedimentation constants obtained for sodium caseinates were found to be reasonably reproducible, whether the casein complex used as starting material was water-washed, serum-washed, or not washed at all (Table 2).

Also shown in Table 2 are the average sedimentation constants of the major components of casein solutions, made by dialyzing sodium caseinate preparations against skimmilk. Blanks in the data for sodium caseinates indicate the absence of the particular components. Two samples of reaggregated colloid were lost because of bacterial contamination. It appears that reaggregation of sodium caseinates by skimmilk dialysis occurs to an extent dependent upon the original particle size of the casein complex fraction used as starting material. After

TABLE 2

Sedimentation constants of sodium caseinates in sodium barbiturate buffer prepared from various calcium caseinate fractions [a]

Casein complex $s_{20,w}$	Sodium caseinates $s_{20,w}$ / Relative concentration [b]				Dialysis aggregate $s_{20,w}$
"A" 980	1.3 / 39	4.4 / 25	6.0 / 25	9.3 / 11	400
870	1.1 / 14	4.3 / 35	5.3 / 49	8.0 / 2	280
640	1.1 / 16	4.2 / 28	5.6 / 34	8.4 / 22	130
230	1.1 / 8	4.0 / 33	5.5 / 49	8.0 / 70	116
"B" 910	2.1 / 28	4.7 / 48	—	10.0 / 24	—
390	2.4 / 34	4.4 / 30	—	9.2 / 36	320
120	1.3 / 45	3.1 / 37	—	8.9 / 18	120
"C" 2,430	1.0 / 21	3.6 / 76	6.6 / 3	—	350
2,240	1.1 / 12	3.3 / 35	4.0 / 3	—	—
1,530	1.2 / 14	3.4 / 86	—	—	230
1,010	1.1 / 14	3.5 / 86	—	—	170
300	1.3 / 14	3.4 / 86	—	—	140

[a] "A" samples were not washed and were dissolved in water. "B" samples were washed with milk serum and dissolved in serum. "C" samples were washed thoroughly with water and dissolved in water.

[b] Relative per cent concentrations corrected for radial dilution.

seven days, the rate of reaggregation was essentially zero. Further dialysis did not measurably change the sedimentation constant of the main component of the aggregate.

It is also seen (in Table 2) that washing of the casein complex with water removed from the system some factor or protein which later appears as the S = 9 to 10 component of sodium caseinate in ultracentrifuge patterns made from unwashed calcium caseinate. If the casein colloid preparation is smaller than S = 2,000, washing with water also removes from the system some factor or protein which appears later as the S = 4 to 6 component in ultracentrifugal patterns of sodium caseinates made similarly.

The return of sodium caseinate particle size to the particle size of the original micelle by reaggregation is most nearly complete in serum-wash experiments, less so in the experiments involving no washing, and least evident in water-wash experiments.

CONCLUSIONS

The distribution of sodium caseinates found in preparations of casein made by replacing calcium with sodium in various size fractions of casein micelles is dependent upon the method of preparation.

Washing casein colloid preparations with water removes some factor or protein causing the loss of sodium caseinate with sedimentation constants of S = 6 and S = 9. Washing casein colloids with milk serum causes the disappearance of a S = 6 component from the resulting sodium caseinate system. It can not be determined from the present data whether missing sodium caseinate components are converted to other components or are preferentially removed from the micelle system during the washing of the deposit.

Sodium caseinates aggregate when dialyzed against skimmilk to a size range dependent upon the size range of the original micelles from which the sodium caseinates were made. Reaggregation of casein is dependent upon factors other than calcium ion concentration alone. That is, the protein itself, once synthesized, or once complexed, retains properties affecting its future aggregation and dissociation.

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